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TITLE OF THE INVENTION

HOOKWORM PLATELET INHIBITOR

BACKGROUND OF THE INVENTION

Field of the Invention. This invention relates to an isolated, purified, and cloned hookworm polypeptide which is a novel potent inhibitor of platelet aggregation and adhesion.

Description of the Related Art. Human hookworms, which currently infect nearly a fifth of the world's population, are a major cause of iron deficiency anemia in developing countries (1-3, 32). Aggressive hematophagous nematodes, hookworms can survive for months to years attached to the intestinal mucosa of their host, chronically sucking blood from small vessels lacerated by sharp teeth or cutting plates (4-6). In children, the effects of longstanding hookworm infection can be severe, and are directly attributable to the debilitating consequences of chronic iron deficiency on physical and intellectual development, often resulting in poor school performance and learning disabilities (7-9). While available anthelminthic agents are effective, re-infection in endemic areas is common (33, 34),

and repeated courses of therapy may be necessary to keep the intensity of infection low and prevent anemia. The results of studies evaluating chemotherapeutic strategies to reduce hookworm anemia have been promising (35, 36), although the ultimate impact on large populations, particularly with regard to drug toxicity in children and pregnant women (32, 37), as well as the emergence of anthelminthic resistance, remains unknown. Although there is no currently available human vaccine for hookworm, recent work in animal models suggests that immunization with recombinant hookworm antigens may be effective at reducing levels of infection following challenge with third stage larvae (38).

Although it has been recognized for nearly a century that hookworms produce potent inhibitors of mammalian thrombosis (10-14), only recently have the molecular mechanisms through which these parasites prevent blood clotting and cause anemia been elucidated. Most of this work has focused on the anticoagulant serine protease inhibitors produced by adult *Ancylostoma caninum* hookworms (15-17, 45). These anticoagulant molecules are members of a family of protease inhibitors first isolated from the non-bloodfeeding intestinal nematode *Ascaris suum* (18-19). To date, two novel inhibitors of coagulation factor Xa and the factor VIIa/tissue factor complex, respectively, have been purified and cloned from *A. caninum* (17, 20).

Some coagulation inhibitors from hookworm have been well characterized, but little is known about how these parasites block platelet function. Previous work by Spellman and Nossel showed that soluble protein extracts of *A. caninum* inhibited platelet aggregation induced by ADP and collagen, which they attributed to an ADP hydrolyzing, or apyrase, activity (13). Later work by Carroll, *et al.*, confirmed the presence of a similar inhibitor of collagen and ADP-induced platelet aggregation in the related hookworm *A. ceylanicum* (14). Interestingly, despite limited evidence, these authors hypothesized that the inhibitor might function by blocking the interaction of collagen with its receptor on the platelet surface. Most recently, studies by Furmidge, *et al.*, using ES products from the hookworm *Necator americanus* characterized broad inhibition of platelet aggregation in

response to multiple agonists, including ADP, collagen, thrombin, and platelet activating factor (39). They theorized that this hookworm species might secrete an inhibitor of GPIIb/IIIa, the platelet surface $\alpha_{IIb}\beta_3$ integrin that mediates adhesion to fibrinogen, as well as activation and aggregation in response to numerous agonists. The molecular mechanism(s) of hookworm platelet inhibition are not understood, and the isolation of the responsible parasite-derived compounds have not been heretofor reported.

BRIEF SUMMARY OF THE INVENTION

It is an objective of the invention to provide a new inhibitor of platelet function useful for medical and veterinary purposes. It is another objective of the invention to provide a potent and specific inhibitor of platelet aggregation and adhesion purified and characterized from adult *Ancyclostoma caninum* hookworms. It is a further and more specific objective of the invention to provide a platelet inhibitor that targets certain cell surfaces, cell surface receptors, and/or adhesion interactions.

These and other objectives are accomplished by the present invention which provides a soluble polypeptide originally isolated and purified from *Ancyclostoma caninum* hookworms, and then cloned. The polypeptide inhibits platelet aggregation and adhesion in response to a variety of agonists, by interfering with the binding of at least one cell surface integrin such as glycoprotein (herein denominated "GP") GPIIb/IIIa ($\alpha_{IIb}\beta_3$) with its respective ligand (fibrinogen for GPIIb/IIIa) and/or the binding of GPIa/IIa ($\alpha_2\beta_2$) with collagen. Thus, the hookworm platelet inhibitor of the invention not only blocks platelet aggregation, it also prevents adhesion of platelets to fibrinogen and/or collagen. A recombinant polypeptide having a predicted molecular weight of about 20.3 to 20.4 kDa is shown in SEQ ID NO: 2. The invention further encompasses fragments or variants thereof exhibiting at least about 50%, and in many embodiments, at least about 65% to about 75%, sequence homology to the naturally occurring polypep-

tide, which exhibit the same biological properties as the native molecule, compositions containing the inhibitor, and methods of using it.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents graphs showing the inhibitory effect of hookworm extracts on platelet aggregation and adhesion. A. Soluble protein extracts of adult *A. caninum* hookworms (30 μg/well) block aggregation of platelets induced by epinephrine (10 μM), thrombin (10 nM), or ADP (10 μM). Open bars: Platelets pre-incubated with hookworm extracts; Closed bars: Platelet controls incubated without extracts. B. Inhibitory effect of hookworm extracts on platelet adhesion to immobilized fibrinogen. Platelets were pre-incubated with increasing amounts of hookworm extracts, then added to fibrinogen (40 μg/mL) coated microtiter plate wells. Adhesion was measured as described in the examples. Results are expressed as percent of control adhesion values, *i.e.*, in the absence of hookworm extracts.

Figure 2 graphs the purification of HPI using anion exchange chromatography. Soluble hookworm extracts from approximately 1000 adult worms was applied to a 25 mL anion exchange column at a flow rate of 2 mL/min. The bound protein (solid line) was eluted with a 0-500 mM NaCl gradient (dashed line). Individual column fractions (5 mL) were collected and tested for inhibition of platelet adhesion to fibrinogen (closed circles).

Figure 3 graphs the purification of HPI using size exclusion chromatography. Approximately 0.5 mL of the concentrated pooled anti-platelet activity from the anion exchange column was applied to a Bio-Sil TSK-125 size exclusion column. Individual column fractions (0.5 mL) were collected and tested for inhibition of platelet adhesion and aggregation. Horizontal bar indicates contiguous fractions containing the HPI activity. Inset: Standard curve of the $\log_{MW}$ vs retention factor (R_f) for the following proteins: thyroglobulin (MW 670 kDa),

gamma globulin (MW 158 kDa), ovalbumin (MW 44 kDa), myoglobin (MW 17 kDa), and vitamin B_{12} (MW 1.3 kDa).

Figure 4 provides bar graphs presenting data on the effect of HPI on platelet aggregation and adhesion. A. HPI (20 μg/well) blocks aggregation induced by epinephrine (10 μM), thrombin (10 nM), and ADP (10 μM). Open bars: platelets pre-incubated with HPI; closed bars: platelet controls. B. HPI blocks platelet adhesion to immobilized fibrinogen and collagen. Platelets were pre-incubated with soluble hookworm extracts (HEX 30 μg/well), HPI (20 μg/well), or monoclonal antibodies 7E3 (5 μg/well), or 6F1 (5 μg/well). Adhesion was measured as described in the examples. Results are expressed a percent of control adhesion detected in the absence of inhibitor.

Figure 5 shows purification of hookworm platelet inhibitor by reversed-phase HPLC as described in the examples. Following anion exchange and size exclusion chromatographies, the partially purified inhibitor was subjected to reversed-phase HPLC using a C_{18} column. The bound protein was eluted under a linear gradient of acetonitrile in 0.1% TFA. Individual peaks of protein were lyophilized, resuspended in aqueous buffer, and tested for inhibition of biotinylated fibrinogen binding to immobilized GPIIb/IIIa and T4 cell binding to collagen.

DETAILED DESCRIPTION OF THE INVENTION

This invention is based upon the finding that a hookworm polypeptide is useful for a number of medical and veterinary therapeutic and prophylactic purposes.

Results reported herein describe the isolation, purification, characterization, and cloning of a protein inhibitor of platelet aggregation and adhesion, hereafter sometimes denominated herein as HPI for "hookworm platelet inhibitor", identified in soluble protein extracts of adult *Ancylostoma caninum* hookworms and

observed in *Necator americanus*. Platelets respond to a variety of agonists, such as, but not limited to, epinepherine, thrombin, and ADP tested in assays described in the examples section. The polypeptide interferes with the interaction of at least one cell surface integrin with its respective ligand, such as GPIIb/IIIa ($\alpha_{IIb}\beta_3$)/fibrinogen or GPIa/IIa ($\alpha_2\beta_1$)/collagen, by binding to the integrin. Preliminary evidence reported herein indicates that HPI probably binds to both. Cloning information predicts a molecular weight of about 20.3 to about 20.4 kDa for a recombinant protein containing amino acid sequences exhibiting functional hookworm platelet inhibiting properties set out in SEQ ID NO: 2. Data reported herein indicate that the molecular weight of active forms of HPI in hookworms have an approximate molecular weight of about 15 to 25 kDa. HPI does not exhibit homology to any other published polypeptide.

By "hookworm" is meant any nematode that sucks blood from the small intestine including, but not limited to, the major hookworms that infect humans, *Ancylostoma duodenale, Necator americanus*, and, less commonly, *A. ceylanicum*, as well as hookworms that infect other animals such as *Ancylostoma caninum*, *Bunostomum phlebotomum, Agriostomum vryburgi, B. trigonocephalum*, and *Gaigeria pachyscelis*. Other blood-sucking nematodes such as *Haemonchus* species, *e.g.*, *H. contortus*, are also encompassed by this invention. *Ancylostoma caninum* is a preferred source.

In the practice of this invention, HPI is isolated and purified from hookworms and then cloned and expressed. By "purified" is meant essentially homogenous, yielding one polypeptide band on electrophoresis in a system that separates proteins; purified inhibitor is thus substantially free of other hookworm constituents, including associated proteins. Generally, the isolation preparation from worms is carried out by homogenizing or lysing the nematodes to obtain soluble extracts, and purifying the protein from the extracts, or obtaining extretory or secretory products from live worms. Cloned proteins are obtained from cultures of respective expression organisms. Any type of protein purification scheme familiar to the skilled artesan can be employed, such as, for example, affinity,

ion-exchange, exclusion, partition, liquid and/or gas-liquid chromatography; zone, paper, thin layer, cellulose acetate membrane, agar gel, starch gel, and/or acrylamide gel electrophoresis; immunochemical methods; combinations of these with each other and with other separation techniques such as dialysis; and the like. One procedure is set out in the examples.

Also encompassed by this invention are synthetic HPI polypeptides exhibiting activity and structure similar to the isolated and purified protein. Typical procedures employ the amino acid sequence of the native polypeptide to prepare cloned complementary DNA sequences defining HPI, *e.g.*, SEQ ID NO: 1, which is then be used to transform or transfect a host cell for protein expression using standard means. Also encompassed by this invention are DNA sequences homologous or closely related to complementary DNA described herein, namely DNA sequences which hybridize, particularly under stringent conditions, to HPI cDNA such as that set out in SEQ ID NO: 1, and RNA corresponding thereto. In addition to HPI-encoding sequences, DNA encompassed by this invention may contain additional sequences, depending upon vector construction sequences, that facilitate expression of the gene. Expressed proteins exhibit properties mimicking native proteins.

DNA starting material which is employed to form DNA coding for HPI of the invention may be natural, recombinant or synthetic. Thus, DNA starting material isolated from tissue or tissue culture, constructed from oligonucleotides using conventional methods, obtained commercially, or prepared by isolating RNA coding for anticoagulant protein, and using this RNA to synthesize single-stranded cDNA which is used as a template to synthesize the corresponding double stranded DNA can be employed to prepare suitable DNA encoding HPI. This DNA, or RNA corresponding thereto, are then inserted into a vector, *e.g.*, a pBR, pUC, pUB or pET series plasmid or baculovirus, and the recombinant vector used to transform a microbial host organisms. Host organisms useful in the invention are bacterial (*e.g.*, *E. coli* or *B. subtilis*), yeast (*e.g.*, *Pichia pastoris* or *S. cervisiae*), insect (*e.g.*, *S. frugiperda*) or mammalian (*e.g.*, mouse fibroblast). This invention

thus also provides novel, biologically functional viral and plasmid RNA and DNA vectors incorporating RNA and DNA sequences describing HPI generated by standard means. Culture of host organisms stably transformed or transfected with such vectors under conditions facilitative of large scale expression of the exogenous, vector-borne DNA or RNA sequences and isolation of the desired polypeptides from the growth medium, cellular lysates, or cellular membrane fractions yields the desired products. Isolation and purification of microbially expressed proteins provided by the invention are by conventional means like that used for the native protein isolations, including chromatographic and immunological separations.

This invention provides compositions containing HPI useful in a number of applications, including, but not limited to, vaccines and pharmaceutical compositions more fully discussed below. The invention encompasses the purified polypeptide shown in SEQ ID NO: 2, or fragments or variants thereof, including variants exhibiting at least about 50%, in many cases from about 65% to about 75%, preferably at least about 80% to about 95%, sequence homology to SEQ ID NO: 2, which inhibit platelet function mimicking the native protein.

HPI of this invention exhibits a number of desirable characteristics. The polypeptide inhibits platelet aggregation and adhesion in response to a variety of agonists, including, but not limited to, ADP, thrombin, and epinepherine. HPI apparently binds to at least one cell surface integrin such as GPIIb/IIIa ($\alpha_{IIb}\beta_3$), and possibly also GPIa/IIa ($\alpha_2\beta_1$), and mixtures thereof. Thus, HPI not only blocks platelet aggregation, it also prevents adhesion of platelets to fibrinogen and possibly also collagen. *In vitro*, the inhibitor inhibits b-fibrinogen binding to GPIIb/IIIa by 80%, which is comparable to a monoclonal antibody used for comparison in one study.

Thus the invention provides platelet inhibiting products for veterinary and medical purposes, particularly in vascular biology and cancer therapy. Platelet adhesion is critical for formation of primary hemostatic thrombi, and is mediated

by adhesion receptors. Under certain circumstances, platelet GPIIb/IIIa receptors on the luminal surface of adherent platelets are activated and undergo a conformational change that results in their binding plasma fibrinogen with high affinity (30). The bivalent structure of fibrinogen allow proteins that bind to GPIIb/IIIa receptors to bind to two or more different platelets simultateously. The GPIIb/IIIa receptor is expressed only in megakaryocytes and platelets, and so is uniquely adapted to its role in platelet physiology. Presumably reflecting the need for an instantaneous response to hemorrhage, the density of GPIIb/IIIa receptors on the surface of platelets is extraordinary ($\sim$80,000 copies spaced $<$ 200Å apart), and there is an additional internal pool of GPIIb/IIIa receptors in α-granules that can be rapidly mobilized to the surface. GPIIb/IIIa ($\alpha_{IIb}\beta_3$) is the most abundant platelet adhesion receptor (46). Agents that are released (*e.g.*, ADP), synthesized and released (*e.g.*, thromoxane A_2), or generated as part of the hemostatic cascade (*e.g.*, thrombin) when vessels are damaged are all able to initiate signals that result in the transformation of the GPIIb/IIIa receptor to a high affinity state. HPI of the invention markedly interferes with binding of fibrinogen to receptor GPIIb/IIIa, and very probably inhibits collagen binding to GPIa/IIa, is a unique target for inhibiting platelet activation in anti-integrin receptor therapeutics. A number of other GPIIb/IIIa antagonists have been suggested and tested for treatment of myocardial infarction and unstable angina, alone, and in combination with aspirin, thrombolytic agents, PCI, stents, and anticoagulants (30). Other conditions in which platelet thrombus formation may contribute to organ damage also benefit from GPIIb/IIIa antagonist therapy, including stroke, cerebral and peripheral arterial angioplasty, thrombotic thrombocytopenic purpura/hemolytic uremic syndrome, heparin-induced thrombosis, microvascular surgery, and cerebral malaria.

Regulation of adhesion molecules has also been suggested for cancer therapy (48). It has been known for some time that cell adhesion receptors, including members of the integrin family, play an important role in the biology of tumors. Until recently, though, most of the research emphasis has been on the study of integrins in the invasive and metastatic behavior of tumor cells. Now it

appears that integrins can function as true receptors capable of transducing signals to the cell interior (48). Thus, integrin inhibition has been suggested for treating a variety of cancers by altering the neoplastic state of tumor cells, repressing gene induction and inhibiting cancer cell migration on extracellular matrix substances and invasion of distant tissues.

Administration of HPI of the invention can be local or systemic. Systemic administration is preferred in some embodiments. Administration can be via any method known in the art such as, for example, oral administration of losenges, tablets, capsules, granules, or other edible compositions; subcutaneous, intravenous, intramuscular, or intradermal administration, *e.g.*, by sterile injections; parenteral administration of fluids and the like. Typical administrations involve the use of the inhibitor dispersed or solubilized in a pharmaceutically acceptable carrier. Local administration is preferred in other embodiments. In these embodiments, the inhibitor, again preferably in association with a pharmaceutically acceptable carrier in which the inhibitor is dispersed or solubilized, is applied in effective amounts directly to an organ. Combinations of therapies may also be employed.

The amount of HPI necessary to bring about the therapeutic treatment is not fixed *per se*, and necessarily is dependent on the concentration of ingredients in the composition administered in conjunction with a pharmaceutical carrier, adjunct compounds in the composition administered that enhance the platelet function inhibitory effect where present, and the age, weight, and clinical condition of the patient to be treated. Preferred compositions deliver the HPI in effective amounts without producing unacceptable toxicity to the patient. Pharmaceutical compositions or formulations of the invention may also include other carriers, adjuvants, stabilizers, preservatives, dispersing agents, and other agents conventional in the art having regard to the type of formulation in question.

In addition, developing strategies aimed at inhibiting the ability of the worm to feed should ameliorate the major clinical effects of this globally important

parasite. Because of their potentially vital role in bloodfeeding, and hence parasite survival, the specific anti-thrombotic molecules produced by adult hookworms represent obvious targets for just such an approach. Moreover, enhancement of an immune response aimed at HPI of the invention provides a vaccine for reducing the burden of hookworm infection in populations at risk. This typically involves immunizing a mammal (animal or human being) by inoculating the mammal with an effective amount of a product prepared by mixing HPI with a suitable carrier such as isotonic saline. Polyclonal, monoclonal, or fusion phage antibodies are then generated using standard means.

The following examples (and reference 49) are presented to further illustrate and explain the present invention and should not be taken as limiting in any regard.

EXAMPLES

Materials and Methods

Reagents and antibodies. Human fibrinogen (plasminogen and von Willebrand factor free) was purchased from Enzyme Research Labs (South Bend, IN). Human α-thrombin was obtained from Haematologic Technologies (Burlington, VT). Type I collagen, bovine serum albumin (BSA), epinephrine, ADP, and sodium citrate were all purchased from Sigma (St. Louis, MO). RPMI media, penicillin, streptomycin, and gentamicin were purchased from GIBCO BRL (Rockville, MD). All other chemicals were of reagent grade. Two inhibitory monoclonal antibodies were employed: 7E3 (21), which blocks platelet aggregation mediated via GPIIb/IIIa ($\alpha_{IIb}\beta_3$), as well as the binding of platelets to immobilized fibrinogen, and 6F1 (22), which blocks the binding of collagen to platelet GPIa/IIa ($\alpha_2\beta_1$).

Platelet preparation. Using a 20 gauge needle, whole blood was drawn by venipuncture from healthy volunteers into one-tenth volume sodium citrate. Following centrifugation at 700 x g for 4.5 minutes, the platelet-rich plasma (PRP) was removed, and the platelet count was adjusted to approximately 100,000/μL

using a Ca^{2+}-free Tyrode's Buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM NaHCO3, 1 mM $MgCl_2$, 5.6 mM glucose, 3.5 g/L BSA). The diluted PRP was kept at room temperature and utilized within 4 hours.

Assay of platelet aggregation. Platelet aggregation was monitored using the microplate method of Bednar, *et al.* (23). Briefly, 100 μL of diluted PRP (100,000 platelets/μL) was added at 25°C to individual wells of a microtiter plate containing 10 μL of ADP (100 μM), human α-thrombin (100 nM) or epinephrine (100 μM). The change in light absorbance in each well (OD_{630}nm/min) was subsequently measured using an MRX HD kinetic microplate reader (Dynex Laboratories, Chantilly, VA). Readings were taken every 20 sec for 6 min, with continuous shaking (14 Hz) between each reading. The rate of aggregation was defined as the change in OD_{630} per min as measured over the 6 min period. Since the OD of a suspension of platelets decreases as aggregation occurs, the results were expressed as absolute values. When PRP was studied without agonist, no spontaneous aggregation, *i.e.*, less than a 10% change in the OD_{630}, was observed over 6 min. All experiments were performed in duplicate, and the values reported represent the average of the two wells.

For assays of aggregation inhibition, 100 μL of diluted PRP was pre-incubated for 10 min at 25°C with 10 μL of hookworm extracts, column fractions, or monoclonal antibody (final concentration of antibody 50 μg/mL). Then 100 μL of the platelet/inhibitor mixture was added to wells of a microtiter plate containing 10 μL of agonist, and the rate of aggregation was measured over 6 min as described above.

Assay of platelet adhesion. Platelet adhesion to fibrinogen, collagen, or BSA was measured in 96-well microtiter plates (24). Individual wells were coated with 150 μL of human fibrinogen (40 μg/mL), type I collagen (20 μg/mL) or BSA (2 mg/mL) for 1 hr at 37°C. The plates were washed 3 times with PBS, followed by the addition of diluted PRP (100,000 platelets/μL) in Ca^{2+}-free Tyrode's buffer. For assays of inhibitory activity, diluted PRP was pre-incubated for 10 min with hookworm protein or monoclonal antibody 7E3 or 6F1 (50 μg/mL). After 10 min, 100 μL of the diluted PRP/inhibitor mixture was added to the coated wells and incubated at 25°C for 90 min. After washing 3 times with PBS,

the adherent platelets in each well were lysed with 100 μL of buffer containing 0.1% Triton X-100, 0.1 M sodium citrate, and 5 mM p-nitrophenyl phosphate (Sigma), a chromogenic substrate for platelet acid phosphatase. After incubation at 37°C for 60 min, the reaction was stopped by the addition of 100 μL of 2 M NaOH. The amount of *p*-nitrophenol produced, corresponding to the amount of intracellular platelet acid phosphatase released, was measured with a microplate reader at 405nm. As a control for non-specific platelet interactions, the values derived for platelet binding to BSA (which was generally less than 10% of the maximum binding detected) were subtracted.

Anion exchange chromatography. A soluble hookworm protein extract (100 mL) was prepared by grinding approximately 1,000 adult *A. caninum* hookworms in a glass homogenizer. The homogenate was resuspended in 50 mM Tris-HCl, pH 8.1, and clarified by centrifugation (15, 16). The soluble protein extract was applied to a 25 mL Spherilose Q-500 anion exchange column (Isco, Lincoln, NE) previously equilibrated with 50 mM Tris-HCl, pH 8.1, at a flow rate of 2 mL/min. After washing, the bound protein was eluted from the column under a gradient of 0-500 mM NaCl. Individual column fractions (5 mL) were collected and tested for inhibition of platelet adhesion and aggregation. The active fractions were pooled and concentrated using a Biomax Ultrafree-4 centrifugal filter (Millipore, Bedford, MA) with a MW cutoff of 5 kDa.

Size exclusion chromatography. A total of 4 mL of the concentrated active fractions from the anion exchange column was applied in 0.5 mL aliquots to a 300 x 7.5 mm Bio-Sil TSK-125 size exclusion chromatography column (Bio-Rad, Hercules, CA) equilibrated with 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl. Individual column fractions (0.5 mL) were tested for inhibition of platelet adhesion and aggregation, and the active fractions were pooled. The MW of the partially purified inhibitor was estimated by extrapolating from a standard curve ($\log_{MW}$ vs R_f) constructed from the elution profile of a mixture of protein standards (Bio-Rad) with known molecular weights (16). The pooled fractions containing the anti-platelet activity were subjected to SDS polyacrylamide gel electrophoresis (SDS PAGE) using a tricine gel buffer system, and individual protein bands were visualized by silver staining (16).

Hookworm excretory/secretory products. Excretory/secretory (ES) products were prepared from live adult *A. caninum* using a method previously described (25) for infective L_3 hookworm larvae. Briefly, adult hookworms were manually harvested from the intestines of laboratory-infected beagles (26) and washed in PBS. Male and female worms were added together in groups of 10 to individual wells of a 24 well tissue culture plate in the presence of 0.5 mL RPMI supplemented with antibiotics (penicillin, streptomycin, and gentamicin) and a low molecular weight (10 kDa) filtrate of canine serum (15% v/v). After incubation overnight at 37°C, 5% CO_2, the worms were removed and the ES products were sterilized using a 0.2 μm centrifugal filter (Millipore). Prior to testing for inhibition of platelet aggregation and adhesion, the ES products were concentrated approximately 5 fold using a spin concentrator with a MW cutoff of 5 kDa. As a control for the platelet inhibition assays, the same culture media (RPMI/antibiotics/serum filtrate) concentrated in a similar manner was used. There was no difference in the total protein concentrations of the two RPMI solutions, *i.e.*, with or without ES, based on analysis using a BCA kit (Pierce, North Rockford, IL).

Fibrinogen binding assay. The binding of b-fibrinogen to immobilized GP IIb/IIIa was measured in an assay that modified the methods from Charo, *et al.* (50) and Steiner, *et al.* (51). Purified GPIIb/IIIa (Enzyme Research Labs.) was diluted to 5 μg/mL in 20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 0.02% NaN_3, pH 7.4 (buffer A) and added to 96-well Immulon 1 microtiter plates (Dynatech Laboratories) at 0.1 mL (0.5 μg) per well. The plates were incubated overnight at 4°C and then washed by filling and emptying by suction with 50 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, 0.02% NaN_3, pH 7.4 (buffer B). Bovine serum albumin (BSA) at 20 mg/mL in buffer B was incubated (0.2 mL/well) for 2 hours at room temperature, for blocking of nonspecific binding. B-fibrinogen was diluted to varying concentrations (1 - 50 nM) in buffer B containing 1 mg/mL of BSA and was incubated in triplicate wells (0.1 mL/well) for 4 hrs. at 37°C. The plates were then washed (x 3) with buffer B before quantifying b-fibrinogen binding. Streptavidin conjugated to horseradish peroxidase (SAV-HRP, Calbiochem) was diluted to 1 μg/mL in buffer B and 0.1 mL was added per well. The plates were incubated for 1 hour at room temperature and then washed (x 3) with buffer B for

removal of unbound SAV-HRP. Peroxidase substrate (1-Step Turbo TMB-ELISA, Pierce) was then added (0.15 mL/well) and changes in light absorbance (OD at 630 nm/min) were measured with a kinetic microplate reader (MRX HD, Dynatech Labs.). Readings were obtained every 30 seconds for 5 minutes, with continuous shaking in between readings. Nonspecific binding was determined by measuring the binding of b-fibrinogen to BSA coated wells and it was generally less than 10% of the maximum binding. The kinetics of b-fibrinogen binding to GP IIb/IIIa were characterized by Scatchard analysis (52).

For an evaluation of inhibitory activity, fibrinogen was incubated at a 10 nM concentration in the presence of various hookworm protein extracts, excretory-secretory (E/S) products and the inhibitory monoclonal antibodies 7E3 and 6F1.

Collagen binding assay. A distinct assay was developed to measure collagen binding to the platelet integrin GP Ia/IIa using T47D mammary carcinoma cells and based on the work by Keely, *et al.* (53). An Immulon 2 microtiter plate was coated with type I collagen in PBS and blocked with BSA as described above. Cells at approximately 90% confluence were detached with versene 1:5000 (0.5 mM EDTA in PBS, Gibco BRL), centrifuged for 5 minutes (4400 RPM), and resuspended in PBS supplemented with 5 mg/mL BSA, 5 mM glucose and 0.3 mM $MgCl_2$. After a second centrifugation, the cells were resuspended and diluted to 500,000 cells/mL and 0.1 mL was added to each well. The plate was incubated for 15 minutes at 37°C and non-adherent cells were removed with low suction and rinsing with PBS three times. Adherent cells were quantified with the Landegren hexosaminidase assay (54) as described by Haugen *et al.* (55). Briefly, after the final rinse with PBS, 60 μL were added to each well of hexosaminidase substrate solution (3.75 mM *p*-itrophenol-N-acetyl-β-D-glucosaminide, 50 mM citrate, and 0.25% Triton X-100). The plate was incubated overnight at 37°C. The color was developed by adding 90 μL/well of 50 mM glycine, 5 mM EDTA, pH 10.4 and absorbance was measured at 405 nm. For testing of inhibitory activity, aliquots of T47D cells at the same concentration were preincubated with hookworm proteins or the monoclonal antibodies in an orbital shaker for 20 minutes at room temperature The cells were then transferred to the designated wells and the assay was

performed as above. Each sample was done in triplicate and nonspecific binding was again determined by measuring binding of cells to BSA coated wells.

Reversed-phase HPLC. A sample of proteins containing HPI was purified from adult *A. caninum* worm extracts through anion-exchange and size exclusion chromatographies as described above. A total of 3 mL (0.8 mg/mL) was applied in 1.0 mL aliquots to a Vydac C18 218TP54 column (0.46 x 25 cm; 5 μm) equilibrated with water in 0.1% trifluoroacetic acid (TFA). The bound protein was eluted over 2 hours with a gradient of acetonitrile (1 - 60%) in 0.1% TFA (Figure 5). Peaks of eluted protein were detected by changes in absorbance at 214 nm and collected individually. After removal of the volatile solvents and resuspension in 20 mM HEPES, each peak was assayed for inhibitory activity against fibrinogen and collagen binding, respectively.

Cloning of the HPI cDNA sequence. Fifty adult *Ancylostoma caninum* worms were resuspended in 1.0 mL of Trizol (Life Technologies) and total RNA was isolated according to the manufacturer's protocol. The RNA pellet was washed with 75% and 100% ethanol, air-dried for 10 min, and resuspended in 40 μL of diethyl pyrocarbonate (DEPC)-treated water.

Degenerate oligonucleotide primers (5'HPI-1 and 3'HPI-1, residues 1 to 23 of SEQ ID NO: 1, and residues 88 to 110 of SEQ ID NO: 1, respectively) were designed based on the amino acid sequence obtained from the native HPI (discussed below). The primers were synthesized by the Keck Foundation Laboratory at Yale. First strand cDNA was synthesized by incubating approximately 1 μg of *A. caninum* total RNA, 10 mM DTT and 100 ng of the degenerate antisense strand primer 3'HPI-1 in first strand reaction buffer (50 mM Tris-HCl, 75 mM KCl, 3mM $MgCl_2$, pH 8.3). The solution was heated for 90 seconds at 90°C and cooled on ice. Each of four deoxynucleotides (1 mM; dATP, dCTP, dGTP, dTTP) was added along with 40 U RNasin (Promega) and 200 U reverse transcriptase enzyme (Superscript II; Life Technologies). This mixture was incubated for 1 hr at 42°C and cooled on ice. The first strand cDNA mixture was combined in thin-walled 0.2 mL tubes with PCR primers 5'HPI-1 and 3'HPI-1 (100 ng of each), PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), and 1 mM of each deoxynucleotide. Taq polymerase (5 U; Perkin-Elmer) was then

added and samples were placed in a thermal cycler (PCR Sprint, Hybaid) for 40 cycles (94°C for 15 sec denaturation, 50°C for 5 sec annealing, 72°C for 30 sec extension), with a final extension at 72°C for 2 min. The reaction mixture was subjected to 1% agarose gel electrophoresis and PCR products were visualized by ethidium bromide staining.

The resulting PCR products were ligated into the pCR2.1 TA cloning vector (Invitrogen) by incubating 10 ng of fresh PCR product, 50 ng of vector, T4 DNA ligase, and ligation buffer overnight at 14°C. One Shot *E. coli* INVαF' cells (Invitrogen) were transformed with the ligation product following the manufacturer's protocol. Samples were plated onto LB agar plates containing 30 μg/mL kanamycin and spread with a 40 mg/mL solution of X-Gal. White colonies that grew overnight at 37°C on the selective media were screened for the appropriately sized insert by direct colony PCR using vector specific primers (T7 Promoter, M13 Reverse), and by restriction enzyme digestion (EcoRI; New England Biolabs) of isolated plasmid DNA. Minipreps (Spin Miniprep Kit, QIAGEN) of positive clones were submitted to the Keck Foundation Laboratory at Yale for nucleotide sequencing.

The sequence obtained was used to design an internal primer (5'HPI-1A, residues 66 to 97 of SEQ ID NO: 1) for a 3' Rapid Amplification of cDNA Ends (3'RACE) protocol used to clone the 3'end cDNA. First strand cDNA was amplified by RT-PCR, as above, using 1 μg of total RNA and an antisense primer, 3'TTTT. The amplified cDNA mixture was then used as a template for PCR using 3'TTTT and 5'HPI-1A. Reaction components were as previously described. Amplification conditions were 40 cycles (94°C for 15 sec denaturation, 55°C for 5 sec annealing, 72°C for 30 sec extension), with a final extension at 72°C for 2 min. The PCR product was ligated into the pCR2.1 TA cloning vector, One Shot *E. coli* INVαF' cells were transformed, and colonies were screened as previously described. Miniprep plasmid DNA of positive clones was submitted to the Keck Foundation Laboratory for nucleotide sequencing.

The full length cDNA was cloned by performing the 3'RACE protocol as described above, with the original 5' end primer (5'HPI-1). The PCR product containing the full sequence was ligated into the pCR2.1 TA cloning vector, One

Shot *E. coli* INVαF' cells were transformed, and colonies were screened as before. The sequence was corroborated by analysis of the Miniprep plasmid DNA at the Keck Laboratory.

Results

Hookworm extracts block platelet aggregation and adhesion. Soluble hookworm extracts blocked platelet aggregation in response to epinephrine, thrombin, and ADP (Figure 1A). Although there was virtually 100% inhibition of aggregation in the presence of 30 μg of hookworm extracts, the effect was found to be dose-dependent for each of the three agonists studied. Because aggregation in response to a variety of agonists is largely dependent on fibrinogen mediated crosslinking of platelets via GPIIb/IIIa, a cell surface $\alpha_{IIb}\beta_3$ integrin (27, 28), these data suggested that soluble hookworm extracts might contain a potent inhibitor of this receptor.

In order to more specifically characterize the anti-platelet mechanism of hookworm extracts, their effect on platelet adhesion to immobilized fibrinogen, which is also mediated via interaction with GPIIb/IIIa (29, 30), were tested. As shown in Figure 1B, hookworm extracts inhibited adhesion of platelets to fibrinogen coated plates in a concentration dependent manner, with 70% inhibition achieved in the presence of 7 μg of soluble hookworm protein.

Hookworm Platelet Inhibitor: partial purification. Soluble hookworm extracts from approximately 1,000 adult *A. caninum* hookworms were first applied to an anion exchange column. The bound protein was eluted with a gradient of 0-500 mM NaCl (Figure 2), and column fractions were collected and tested for anti-platelet activity. No inhibition of platelet aggregation or adhesion was observed in the flow-through fractions. A single peak of anti-platelet activity was identified in the protein eluted from the column, with complete overlap of the fractions inhibiting aggregation and adhesion to fibrinogen. These five fractions, which contained all of the anti-platelet activity present in the pre-column material, were pooled and concentrated.

Following anion exchange, the partially purified inhibitor was then subjected to size exclusion chromatography. Individual fractions were collected and tested for activity using both the adhesion and aggregation assays. Again, all of the anti-platelet activity eluted in a single series of contiguous fractions (Figure 3), which were shown to inhibit aggregation (using ADP as agonist), as well as adhesion to fibrinogen. Based on extrapolation from a standard curve constructed using proteins of known molecular weights (Figure 3, inset), the size of the partially purified active HPI was estimated to be approximately 15 kDa.

SDS PAGE of the partially purified HPI revealed multiple protein bands detected by silver staining, ranging in estimated MW from 10-25 kDa. There was no obvious enrichment of any single band visible by silver staining when HPI was compared to SDS PAGE of the soluble hookworm extract starting material. However, based on measurements of protein concentration and percent inhibition of platelet aggregation, the HPI activity was estimated to have been enriched by approximately 5-fold after anion exchange and size exclusion chromatographies.

HPI: dual inhibition of GPIIb/IIIa and GPIa/IIa. In order to confirm that the purified inhibitor of platelet function contained the activity present in soluble whole worm extracts, the active fractions from size exclusion chromatography were pooled and again characterized with regard to their inhibitory properties. As shown in Figure 4A, partially purified HPI exhibited a comparable inhibitory profile to soluble hookworm extracts, effectively blocking platelet aggregation in response to epinephrine, thrombin, and ADP.

In order to more fully characterize its spectrum of inhibition of platelet function, the effect of HPI on adhesion to collagen, which is mediated via GPIa/IIa (31), an $\alpha_2\beta_1$ integrin distinct from GPIIb/IIIa ($\alpha_{IIb}\beta_3$), was evaluated. As shown in Figure 4B, partially purified HPI also blocked platelet adhesion to collagen. The specificity of the adhesion assay was evaluated by performing control experiments using inhibitory monoclonal antibodies to GPIIb/IIIa (7E3) and GPIa/IIa (6F1). As shown in Figure 4B, these experiments confirmed that the

assay differentiated between inhibition of each individual receptor, since antibody 7E3 only blocked platelet binding to fibrinogen, while 6F1 inhibited only collagen binding. Importantly, an identical spectrum of inhibition of platelet adhesion was seen with soluble hookworm extracts, the starting material for our purification of HPI (Figure 4B). Subsequent evaluation of column fractions from both anion exchange and size exclusion chromatography revealed complete overlap, *i.e.*, co-purification, of the two activities. Though subsequently separated using reversed-phase HPLC as described above, preliminary evidence shows that the fractions are identical or very closely related.

The function of HPI was further characterized using the specially developed fibrinogen and collagen binding assays described above. HPI inhibited fibrinogen binding to GP IIb/IIIa by approximately 80%, which is comparable to the monoclonal 7E3. Inhibitory activity was also detected in E/S products, as well as in extracts from *A. ceylanicum* and *N. americanus* (see below). In the collagen binding assay developed, HPI inhibited T47D cells binding to collagen by approximately 80-85%, comparable to the monoclonal 6F1.

HPI is secreted by adult *A. caninum*. In order to gain potential insight into the biological significance of HPI purified from whole hookworm extracts, ES products from live adult hookworms removed from an infected dog were tested. As shown in Table 1 below, concentrated hookworm ES products contain an inhibitor of platelet aggregation and adhesion that is comparable to that which was purified from soluble extracts of whole worms. Similarly, concentrated culture media, which was comparable to hookworm ES in terms of protein concentration, did not inhibit platelet aggregation or adhesion *in vitro*. These data confirm that the adult hookworm secretes a compound with an identical spectrum of anti-platelet activity to HPI, strongly suggestive of a biologic role for the inhibitor *in vivo*.

Table 1. Adult *A. caninum* excretory and secretory products (ES) inhibit platelet aggregation and adhesion.

	Aggregation (%)	*Adhesion (%)*	
		Fibrinogen	*Collagen*
Control	100	100	100
RPMI	98	104	102
ES	11	58	14

(Note: Results are expressed as percentage of control values for both assays. Aggregation was measured in response to agonist ADP. ES products and RPMI culture media were both concentrated ~5-fold prior to use.)

Hookworm Platelet Inhibitor: native protein analysis, cloning and sequencing. The partially purified HPI described above was subjected to reversed-phase HPLC using a C_{18} column. Bound protein was eluted under a linear gradient of acetonitrile in TFA as described above and shown in Figure 5. Individual protein peaks were lyophilized, resuspended in aqueous buffer, and tested for inhibition of biotinylated fibrinogen to immobilized GPIIb/IIIa. The two activities were distinctly separated. Protein peaks 26 and 27 (designated HPI-2) exclusively inhibited collagen binding to GP Ia/IIa while peaks 28, 29 and 30 (designated HPI-1) inhibited fibrinogen binding to GP IIb/IIIa.

Protein analysis of the purified HPI-1 and HPI-2 was performed at the William Keck Foundation Biotechnology Resource Laboratory at Yale School of Medicine. The molecular mass was determined by electrospray ionization mass spectrometry (ESMS) using a Micromass Q-Tof spectrometer and estimated to be approximately 20,314 - 20,365 Da for HPI-1, and approximately 20,546 Da for HPI-2. HPI-1 was then submitted to NH_2-terminal amino acid sequencing using an Applied Biosystems sequencer equipped with an on-line HPLC system. The first 40 amino acids were obtained (residues 1 to 40 of SEQ ID NO: 2), and the sequence used to design degenerate oligonucleotide primers for cloning of the cDNA corresponding to the HPI-1 gene.

Further analysis of HPI-2 suggests that in fact it could be the same protein as HPI-1, or alternatively, that the two are very closely related. NH_2-terminal amino acid sequencing of HPI-2 revealed that the first six amino acids were identical to those of HPI-1. An internal segment of each protein was sequenced after trypsin digestion, and the amino acid sequence was also identical.

NH_2-terminal sequence:

HPI-1: Glu-Gly-Asp-Tyr-Ser-Leu

HPI-2: Glu-Gly-Asp-Tyr-Ser-Leu

Internal sequence after trypsin digestion:

HPI-1: Thr-Ser-Asn-Ile-Ala-Asn-Met-Val

HPI-2: Thr-Ser-Asn-Ile-Ala-Asn-Met-Val

The NH_2-terminal amino acid sequence of recombinant HPI isolated and analyzed as described above is set out in residues 1 to 40 of SEQ ID NO: 2. Degenerate oligonucleotide primers designed from this sequence were used to obtain the cDNA sequence set out in SEQ ID NO: 1 using cloning procedures described above. The full amino acid sequence comprising residues 1 to 40 of the purified native protein and deduced residues 41 to 181 of the cDNA is set out in SEQ ID NO: 2. The predicted molecular weight of this recombinant polypeptide is 20,333 Da, and its theoretical isoelectric point, 4.76. The estimated molecular weight of native HPI is about 20,314 to 20,365 Da. The HPI sequence was compared to other GENBANK sequences (published at www.ncbi.nlm.nih.gov), and found to exhibit little or no homology to previously published sequences.

Discussion

The data reported herein confirm earlier observations that *Ancylostoma* hookworms produce a potent and broad spectrum inhibitor of platelet aggregation. Also, at least with regard to inhibition of aggregation, the activity present in *A. caninum* is similar to that identified in the phylogenetically distinct hookworm species *N. americanus*. Importantly, an HPI-like activity in ES products of live adult hookworms removed from the gut of a permissive host (dog) was identified, strongly suggesting that secretion of the hookworm inhibitor is associated with the

bloodfeeding process. Preliminary data suggest that the activity is also present in secretory products of infective third stage larvae, suggesting a potentially broad functional role for HPI in the biology of hookworm.

While it is intriguing to speculate that the anti-platelet activity identified from extracts and ES products of adult *A. caninum* hookworms is attributable to a single, multi-functional inhibitor, it remains a possibility that the inhibition of GPIIb/IIIa ($\alpha_{IIb}\beta_3$) and GPIa/IIa ($\alpha_2\beta_1$) is, in fact, due to at least two related inhibitors with distinct molecular targets. Such a scenario would closely resemble that previously encountered with the hookworm inhibitors of factor Xa (AcAP5) (15, 16) and factor VIIa/tissue factor (AcAPc2) (17), whose anticoagulant activities co-purified through multiple chromatographic steps. A variety of diverse hematophagous species, including leeches (40, 41), ticks (42, 43), and biting flies (44), produce potent inhibitors of platelet binding to fibrinogen. Preliminary experiments with purified HPI, though, indicates that the fractions are either identical or very closely related.

The above description is for the purpose of teaching the person of ordinary skill in the art how to practice the present invention, and it is not intended to detail all those obvious modifications and variations of it which will become apparent to the skilled worker upon reading the description. It is intended, however, that all such obvious modifications and variations be included within the scope of the present invention, which is defined by the claims that follow. The claims are intended to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates the contrary.

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The papers cited herein are expressly incorporated in their entireties by reference.